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# Preparation of Copepods for Histopathological Examinations

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# PREPARATION OF COPEPODS FOR HISTOPATHOLOGICAL EXAMINATIONS

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# PREPARATION OF COPEPODS FOR HISTOPATHOLOGICAL EXAMINATIONS[1]

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BARSZCZ, C. A. & YEVICH, P. P. 1976. Preparation of copepods for histopathological examination. *Trans. Amer. Micros. Soc.*, 95: 104–108. Various fixatives and processing techniques were utilized to determine the best method of preparing large numbers of copepods for histopathological examination. Dietrich's fixative gave the finest cytological detail and was the best suited for general use.

Few reports have been published on the preparation of copepods for microscopic study using standard histopathological techniques. Howes et al. (1972) used electron microscopy techniques for their histological studies. However, these techniques are too time consuming for laboratory and field studies involving large numbers of animals.

This paper presents the results of several experiments involving fixatives, fixation time, use of a decalcifying agent, time in the dehydrating and clearing reagents and in paraffin. A technique to facilitate handling the animals was also developed.

## MATERIALS AND METHODS

The species of copepods examined histopathologically in our laboratory included *Acartia clausi*, *A. tonsa*, *Eurytemore affinis*, *Tortanus discaudatus*, *Temora longicornis*, *Centropages typicus*, *Pseudocalanus minutus*, *Calanus finmarchicus*, and *Tigreopus japonicus*. Fifty to 500 live animals were put into each of the following fixatives: Zenker's, Helly's, (zinc chloride was substituted for mercuric chloride in both of the preceding formulas (Jones, 1964), Carnoy's, Bouin's, Dietrich's, 10% buffered formalin, absolute alcohol, and osmium tetroxide. A few drops of eosin were added to the fixatives to make the animals more visible.

The first series of experiments, using the above fixatives, indicated that some of them would not be useful for the routine processing of these animals. Dietrich's, Zenker's, and Helly's were chosen as the fixatives showing the most promise. In an effort to improve the quality of Helly's and Zenker's fixatives, the concentration of formalin in Helly's and acetic acid in Zenker's was varied from 1–10%. Helly's fixative was also made with sea water. None of these variations proved advantageous.

The next series of experiments involved the amount of time the copepods remained in fixative. It was determined that 10 min was the maximum time allowable in Zenker's or Helly's fixatives. Longer periods of fixation caused the animals to become hard and brittle, with shattering of the tissue during sectioning. Animals were left in Dietrich's fixative for several days with minimal damage to the tissues.

In an attempt to reduce the shattering of the exoskeleton during sectioning, Decal[2] was added either directly to Helly's and Zenker's fixatives or was added to the container of animals after the fixative was decanted. Neither method im-

[1] We wish to thank Dr. Juan Gonzalez for collecting and identifying the animals for us.
[2] The use of Decal, the Harleco decalcifying reagent, does not imply endorsement by the Federal Government.

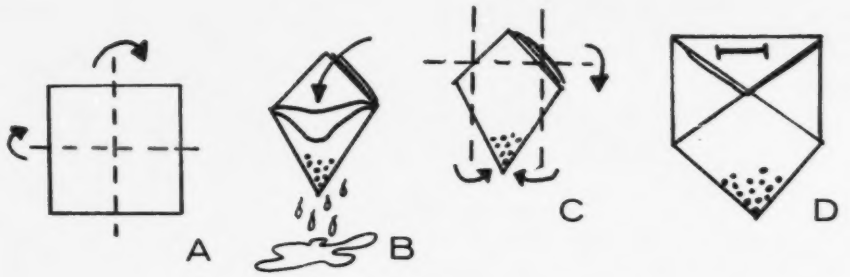



FIG. 1. Nylon net envelope. A. 5 cm square nylon netting. Fold on dotted lines. B. Open to form funnel; pour in copepods. C. Fold in corners; fold down top on dotted lines. D. Staple top of envelope.

proved the quality of the sections. However, leaving the copepods in Dietrich's for two or three days softened the exoskeleton enough to prevent shattering. The glacial acetic acid in Dietrich's acted as the decalcifying agent.

Several types of containers were utilized to carry the copepods through the processing reagents. These include lens paper, nylon netting envelopes, and fine mesh wire baskets. It was found that envelopes of the 50-$\mu$m mesh nylon netting were the most satisfactory. Up to 25 animals were enclosed in each envelope. If too many copepods were included in each envelope, they had a tendency to stick together, which prevented proper infiltration by the paraffin.

Processing time was considerably reduced when it was determined that 10 min in each container was sufficient for the dehydrating, clearing, and infiltrating reactions to take place. The time in the paraffin was the most critical. If the animals remained in the paraffin baths too long, they became shrunken, hard, brittle, and sectioned poorly. Microscopically, the animals showed shattering of the tissue and very poor staining characteristics. Because the embedding procedure was relatively lengthy, it was found advisable to process no more than six cassettes at one time to keep exposure to hot paraffin at a minimum.

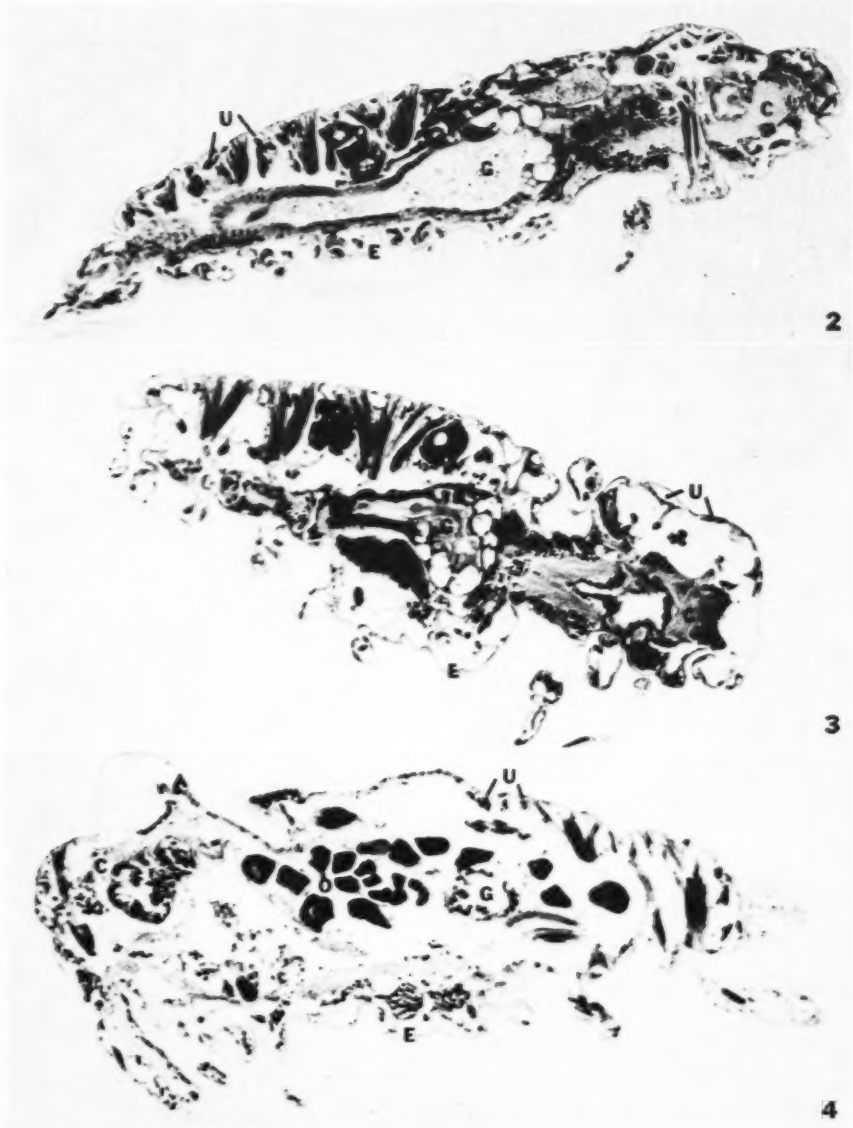
After the copepods were embedded, the blocks were serial-sectioned on a rotary microtome at 5 $\mu$m. Half of the slides were stained with Harris' hematoxylin and eosin-Y and the other half with various stains, such as a modified Movat's Pentachrome stain or Heidenhain's aniline blue method for connective tissue (Luna, 1968).

## RESULTS

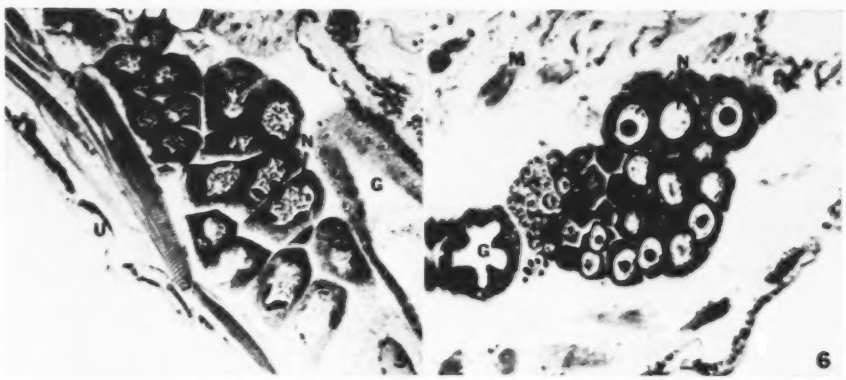
These experiments resulted in the following method for the preparation of copepods for histopathological examination. Dietrich's became the fixative of choice. Following fixation, the copepods were enclosed in small envelopes of 50-$\mu$m mesh nylon netting. These were made by cutting the netting into 5 cm squares, folding the square into quarters, and opening it to form a funnel. The animals were poured into the funnel. The sides of the funnel were gently folded toward the center and the top folded down and stapled to form a triangular envelope (Fig. 1).

The envelopes were placed in cassettes, washed in running water for 16–22 hr, then stored in S-29[3]. The animals were dehydrated, cleared, and infiltrated

[3] The use of S-29 and UC-670, Technicon Corporation dehydrating and clearing reagents, does not imply endorsement by the Federal Government.




FIGS. 2–4. *Acartia tonsa.* Fig. 2. Dietrich's fixative, sagittal section, H & E. × 180. Fig. 3. Helly's fixative for 10 min, sagittal section, H & E. × 180. Fig. 4. Formalin fixed for 20 min, sagittal section, H & E. × 180. Legend: O, ova; C, circumesophageal ganglion; M, muscle; G, gastrointestinal tract; E, exoskeleton; N, nuclei of ova; U, unicellular glands.




FIGS. 5, 6. *Acartia tonsa*. Fig. 5. Helly's fixative for 10 min, H & E. Nucleoplasm present in ova. × 460. Fig. 6. Bouin's fixative for 20 min, H & E. Note loss of nucleoplasm in ova. × 460.

with paraffin on an automatic tissue processor on the following schedule: 10 min in each of six beakers of S-29, 10 min in each of two beakers of UC-670[3], and 10 min in each of two paraffin baths. The same schedule can be used for hand processing tissues. When other reagents are used, the correct processing schedule will have to be determined empirically.

When infiltration was completed, the envelope was removed from the cassette, the stapled end cut off, and the opened square inverted into a small embedding mold partially filled with melted paraffin. The animals normally dropped off the netting and settled to the bottom of the mold. Sometimes it was necessary to rub gently on the top surface of the netting with a warm teasing needle to remove the copepods. The animals were allowed to settle at random to provide a variety of sections.

After the blocks had cooled, they were serial-sectioned and stained as desired.

## DISCUSSION

Dietrich's fixative fulfilled our criteria of a good fixative for the routine preparation of copepods (Table 1). The animals could be held in Dietrich's indefinitely without adverse effects, making it well suited for use on research vessels and field trips. The copepods sectioned well and the cellular detail was excellent (Fig. 2).

Zenker's and Helly's fixatives provided good cellular detail (Fig. 3), but because of the danger of over fixation, they were considered less adaptable to our needs.

Ten per cent buffered formalin gave the poorest results (Fig. 4). The animals were hard, brittle, sectioned poorly and showed very poor cytological detail and poor staining qualities.

Bouin's fixative gave good cytological detail. However, microscopical examination of animals which were fixed in fixatives containing glacial acetic acid (Bouin's, Zenker's, and Dietrich's) showed a loss of nucleoplasm in the ova, the loss being the most extensive in Bouin's fixed animals (Fig. 6). This was not observed in the nuclei of the ova of animals fixed in 10% buffered formalin or Helly's (Fig. 5).

TABLE I
Results of fixation

| Fixative | Length of fixation | Cellular detail | Shrinkage | Brittleness | Staining characteristics |
|---|---|---|---|---|---|
| Dietrich's | 5 min – indefinitely | Excellent | Very little | None | Good |
| Bouin's | 20 min | Good | Very little | None | Fair |
| Carnoy's | 20 min | Fair | Very little | None | Poor |
| 10% Buffered Formalin | 20 min | Poor | Extensive | Yes | Poor |
| Helly's | 10 min | Good | Little | None | Excellent for Heidenhain's & Movat's Stains |
| Zenker's | 10 min | Good | Little | None | Good |

Satisfactory histochemical staining for glycogen and fat was achieved when absolute alcohol and osmium tetroxide were used, but these fixatives were not suitable for routine use because of the artifacts they caused in the tissues.

The methods described in this paper have been used successfully with many small marine crustaceans and small fish larvae. They are also useful when working with delicate tissues such as the ganglions of molluscs and the nervous system of lobsters.

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